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Study

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13. ABSTRACT (Maximum 200 Words)

In a case-control study, nested within a cohort of 4,888 women with BBD, we demonstrated that p53 protein accumulation detected by immunohistochemistry was associated with a 2.5-fold increase in the risk of subsequent breast. However, by using immunohistochemistry alone, we may have underestimated the true risk of developing breast cancer. We hypothesized that p53 mutations in benign breast tissue are associated with increased risk of subsequent breast cancer. We are testing our hypothesis by:
(1) analyzing benign breast tissue from 138 cases and 556 controls for the presence of p53 mutations using PCR-SSCP and PCR-direct DNA sequencing; and (2) estimating the risk of breast cancer in relation to: (a) the presence of p53 mutations in BBD; and (b) the presence of both p53 mutations and p53 protein accumulation in BBD.

DNA was extracted fro theparaffin-embedded breast tissue and exons 2 to 11 of p53 were screened by PCR-SSCP followed by manual sequencing if an abnormality was detected. Manual sequencing was used as we found it detects more gene alterations than GeneChip[R] array. Women with an alteration in intron that is not a polymorphism and positive immunostaining for p53 have a 2.9 fold increased risk to develop breast cancer.

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INTRODUCTION

Our work has been directed towards identifying genetic and protein changes in benign breast disease (BBD) that might be involved in the pathogenesis of breast cancer, and which might serve as markers of risk. We have demonstrated that p53 protein accumulation detected by immunohistochemistry was associated with a 2.5-fold increase in the risk of subsequent breast cancer in a case-control study, nested within a cohort of 4,888 women with BBD (1). These women (4,888) were enrolled in the National Breast Screening Study (NBSS) and had received a histopathologic diagnosis of benign breast disease during the active follow-up phase of the NBSS (2,3). The NBSS is a multi-center randomized controlled trial of screening for breast cancer in 89,835 Canadian women who were recruited between 1980 and 1985, and who were followed actively until 1988 and passively thereafter. Women were eligible to participate if they were 40-59 years old and had no previous history of breast cancer (in situ or invasive).

However it is possible that by using immunohistochemistry alone, we may have underestimated the true risk of developing breast cancer in association with p53 changes. It has been shown that approximately 33% of p53 mutations do not alter the protein in such a way that there is positive immunostaining (4-7). Therefore, a more complete assessment of the role of the association between p53 and breast cancer risk will come from studies combining both immunohistochemistry and p53 gene sequencing. We hypothesized that p53 mutations in benign breast tissue are associated with increased risk of subsequent breast cancer.

In this project we tested our hypothesis by:
(1) analyzing benign breast tissue from 138 cases and 556 controls for the presence of p53 mutations using PCR-SSCP followed by manual DNA sequencing; and
2) estimating the risk of breast cancer in relation to: (a) the presence of p53 mutations in BBD; and (b) the presence of both p53 mutations and p53 protein accumulation in BBD. We will localize the mutations to determine whether they occur preferentially in specific sites of the DNA and to compare them to known mutations listed in p53 mutation banks (8).

BODY

In order to begin this work we first had to design primers for exons 2 to 11 of the p53 gene that would be suitable to use to analyze DNA extracted from paraffin-embedded breast tissue. Exon 4 is a larger exon and we had difficulty reliably obtaining a PCR product because of the nature of DNA obtained from paraffin embedded tissue. To circumvent this we had to identify and develop the conditions for additional primers that would generate PCR products of smaller sizes. These are also included in table 1. The next series of experiments were designed to identify the optimal PCR conditions, e.g. temperature, cycle number, primer concentration, and magnesium concentration, for each exon. The resulting conditions are also summarized in table 1.

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Table 1: PCR Primers for p53 GeneChip[R] Analysis

Exon	Primers*	PCR Product Size
2	5'-TCATGCTGGATCCCCACTTTTCCTCTTG-3' 5'-TGGCCTGCCCTTCCAATGGATCCACTCA-3'	162
3	5'-AATTCATGGGACTGACTTTCTGCTCTTGTC-3' 5'-TCCAGGTCCCAGCCCAACCCTTGTCC-3'	88
4	5'-GTCCTCTGACTGCTCTTTTCACCCATCTAC-3' 5'-GGGATACGGCCAGGCATTGAAGTCTC-3'	366
5	5'-CTTGTGCCCTGACTTTCAACTCTGTCTC-3' 5'-TGGGCAACCAGCCCTGTCGTCTCTCCA-3'	270
6	5'-CCAGGCCTCTGATTCCTCACTGATTGCTC-3' 5'-GCCACTGACAACCACCCTTAACCCCTC-3'	202
7	5'-GCCTCATCTTGGGCCTGTGTTATCTCC-3' 5'-GGCCAGTGTGCAGGGTGGCAAGTGGCTC-3'	173
8	5'-GTAGGACCTGATTTCCTTACTGCCTCTTGC-3' 5'-ATAACTGCACCCTTGGTCTCCTCCACCGC-3'	239
9	5'-CACTTTTATCACCTTTCCTTGCCTCTTTCC-3' 5'-AACTTTCCACTTGATAAGAGGTCCCAAGAC-3'	144
10	5'-ACTTACTTCTCCCCCTCCTCTGTTGCTGC-3' 5'-ATGGAATCCTATGGCTTTCCAACCTAGGAAG-3'	208
11	5'-CATCTCTCCTCCCTGCTTCTGTCTCCTAC-3' 5'-CTGACGCACACCTATTGCAAGCAAGGGTTC-3'	223

* as provided by the manufacturer

To perform the PCR-SSCP and sequencing, 5um sections were cut from the paraffin blocks, dewaxed and stained briefly in hematoxylin. The breast epithelium was microdissected out using a blade, collected in a microfuge tube and digested with proteinase K (GIBCO BRL, ON, 0.5 mg/mL in 50 mM Tris HCl, pH 8.5, 10 mM EDTA, 0.5% Tween 20) for at least 48 hours at 55°C.The proteinase K was inactivated by heating to 95°C for 15 minutes. An aliquot of the digest was amplified using PCR, [α-^{33}P]-dATP and exon specific primers. An aliquot of the reaction product was separated on an 8% non-denaturing polyacrylamide gel and the gel was then processed for autoradiography. Each sample was run under two conditions (2 and 10% glycerol in the loading buffer). Potential mutations were identified as shifts in band mobility. Samples that showed an abnormal band migration in either one or both gels (please see a representative SSCP gels in Figures 1 & 2) underwent repeat PCR-SSCP in the presence of the appropriate percent glycerol. If no shift was seen then the sample was considered wild type for that exon. If two different patterns were seen in the two PCR-SSCP gels then another tissue section was cut, microdissected and underwent PCR-SSCP. If the band shift was confirmed, the band was excised from the SSCP gel and the DNA eluted into water. The DNA was reamplified by PCR using the same primers and the product run in a 2% agarose gel. The band was excised and the DNA eluted using QIAquick gel extraction kit. The purified DNA was manually sequenced using the the Thermosequenase radiolabelled terminator cycle sequencing kit and the sense primer. This was followed by electrophoresis on a 6%, 8.3M urea, denaturing polyacrylamide gel and autoradiography. To confirm the mutation in some samples the DNA was sequenced using the anti-sense primer. Negative controls were included as well as DNA obtained from cell lines with known mutations in p53 where appropriate. The results from each sequencing reaction was compared to the p53 sequence provided by the International Agency for Cancer Research (IARC) database (http://www.iarc.fr/p53)(8).

A total of 189 gene alterations were detected in 462 subjects (which was the number of tissue samples suitable for analysis and provided sufficient DNA) (Figure 1 and 2). Of these 462 subjects 54, (11.7%) had mutations that resulted in an amino acid change; 31 subjects (6.7%) had silent alterations; 33 (7.1%) had intronic sequence changes. Thirty-six subjects had more than one change. The commonest p53 alteration was a mutation causing an amino acid change (38.6% of all alterations).

A total of 131 polymorphisms were detected in 107 subjects. Seventeen subjects had more than one polymorphism. The polymorphisms occurred in exon 4 (codon 72), or 6 (codon 213), intron 2 (G→C), intron 3 (16bp insertion), or intron 9 (T→C). The commonest polymorphism was the 16 base insertion.

These were analyzed for case-control frequency. Odds ratios (OR) and 95 percent confidence intervals (CI) for the associations between p53 gene changes and risk of breast cancer were obtained from conditional logistic regression models (9). Adjusted odds ratio estimates were obtained by including terms representing the following potential confounders in the regression models: history of breast cancer in a

first degree relative, age at menarche, age at first live birth, menopausal status (pre-, peri-, and post-menopausal), body mass index (weight(kg)/height(m)2), and hyperplasia (ductal or lobular, with or without atypia). (Women who reported having had a menstrual period within the last year were defined as premenopausal, as were those who had had a hysterectomy without bilateral oophorectomy and were less than 45 years of age; those who had ceased having menstrual periods within the last 12 months without surgical intervention were defined as postmenopausal, as were those who had had a bilateral oophorectomy and those who had had a hysterectomy only and were more than 55 years of age; the remaining women were classified as perimenopausal.) For categorical variables, tests for trend (on one degree of freedom) in associations were performed by fitting the categorized variables as continuous variables in conditional logistic regression models. All statistical tests were two-sided.

The data suggests that a gene change in an intron that was not a polymorphism was associated with a 2.7fold increased risk to develop breast cancer but the confidence intervals were relatively wide so this observation was not significant (Table 2). However when this change was analyzed together with the presence of immunopositivity for p53 the odds ratio was 2.9 with confidence intervals of (1.1–7.75) for breast cancer risk.

TABLE 2 Risk of breast cancer in association with p53 gene changes in benign breast tissue

p53 change	Odds ratio (95% CI)*
Any change	1.13 (0.62-2.08)
Any change in an exon	0.92 (0.50-1.71)
Any change in an intron	1.43 (0.73-2.82)
Any change in exon/intron that is not a polymorphism	1.55 (0.78-3.05)
Any change in exon/intron that is not a polymorphism – excluding unreadable changes	1.63 (0.79-3.37)
Any change in exon that is not a polymorphism	1.24 (0.59-2.59)
Any change in intron that is not a polymorphism	2.71 (0.89-8.21)
Any change in intron (not polymorphism) and positive for p53 immunoreactivity	2.93 (1.11-7.75)
Any change in exon that leads to amino acid change – all such changes	0.90 (0.48-1.70)
All polymorphisms	0.75 (0.36-1.55)
Exon polymorphisms	0.61 (0.24-1.58)
Intron polymorphisms	1.00 (0.44-2.31)

* Adjusted for age at menarche, age at first live birth, menopausal status, Quetelet's index, family history of breast cancer in a first degree relative, epithelial hyperplasia.

An alternative method to evaluate the p53 gene is the p53 GeneChip[R] (Affymetrix), which utilizes oligonucleotide microarray technology to detect mutations (10). The chip contains over 50,000 oligonucleotide probes, each of which is 18 nucleotides in length and synthesized using light-directed combinatorial chemistry (11). The probes were created to screen the sense and antisense strands of exons 2 to 11 for missense mutations, single base deletions, and the splice sites of the human p53 coding sequence. The p53 GeneChip[R] has been compared with direct sequencing for identifying p53 gene alterations in DNA extracted from frozen tissue of 108 ovarian cancers. The p53 GeneChip[R] had a 94% accuracy rate, 92% sensitivity and 100% specificity compared to 87% accuracy, 82% sensitivity and 100% specificity for direct sequencing (12). In another study the p53 GeneChip[R] was also shown to be comparable to direct sequencing when DNA was extracted from frozen tumour tissue or blood (13). However, its ability to detect p53 gene alterations in DNA that has been extracted from formalin-fixed paraffin embedded (FFPE) tissues is not known. A recent study using arrayed primer extension microarray suggested that it might be possible to assess DNA extracted from FFPE by microarray (14). The purpose of this part of the study was to determine whether the p53 GeneChip[R] could be used to sequence the p53 gene in DNA extracted from formalin-fixed, paraffin- embedded breast tissue. As this methodology might require less DNA than PCR-SSCP and as it is less labour intensive, it might be more appropriate to use than manual sequencing.

Aliquots of DNA, obtained from proteinase K digests of breast cancer samples as described above, were purified using a MiniElute Agarose Gel Purification kit (Qiagen, ON) according to the manufacturer's protocol. The sample was eluted in 10 to 15 µL depending on the amount of tissue that had been microdissected. The DNA was amplified in a multiplex PCR reaction as recommended by the manufacturer and the primers are listed in Table 1. Each 100 µL PCR reaction included 1X PCR buffer (PE Biosystems, MA) 2.5 mM $MgCl_2$, (PE Biosystems, MA) 0.2 mM each of dATP, dCTP, dGTP and dTTP (Eppendorf, NY), 1X p53 GeneChip primer set (Affymetrix, Santa Clara, CA) and 0.8 U of Amplitaq Gold (PE Biosystems). The PCR was performed using a PE 9600 thermal cycler. A 5 µL aliquot of the multiplex PCR was visualized on a 10% polyacrylamide gel to confirm amplification of 10 PCR products of the correct size. These were then fragmented using DNase I, and labelled using fluorescein-ddCMP. Each 50 µL fragmentation reaction included 45µL of the multiplex PCR reaction, 0.005U fragmentation reagent (DNase I in 10 mM Tris HCl (pH 7.5), 10mM $CaCl_2$, 10 mM $MgCl_2$, 50% glycerol) (Affymetrix), 0.03mM EDTA, 0.05 U Calf Intestinal Alkaline Phosphatase (Roche, QB) and 0.5 mM Tris acetate, pH 8.2. The reaction was incubated for 15 minutes at 25°C followed by heat-inactivation of the enzyme at 95°C for 10 minutes. To confirm the fragmentation, a 5 µL aliquot of the sample was visualised in a 2% agarose gel, which showed collapse of the 10 PCR products to fragments of approximately 50 base pairs.

Each terminal labelling reaction contained 50 µL of the amplified and fragmented target, 1X reaction buffer (Enzo Diagnostics,NY), 1X $CoCl_2$ (Enzo Diagnostics), 1X fluorescein-ddCTP (Enzo Diagnostics), and 1X terminal deoxynucleotide transferase

(Enzo Diagnostics, NY). The reaction was incubated at 37°C for 45 minutes and 5 µL of 0.2M EDTA was added to stop each reaction. To confirm the labelling of the multiplex PCR product a 3 µL aliquot of the sample was visualised on 2% agarose gel (UVP Gel DocSystem, CA). DNA was hybridized to the p53 GeneChip[R], washed and scanned (GeneChip Microarray Facility, Albert Einstein College of Medicine). The data analysis was performed using the Affymetrix Microarray Suite to generate a score for each sample. A score of ≥ 12 was considered indicative of a gene alteration. When an alteration detected by the p53 microarray was not confirmed by manual sequencing, the DNA underwent repeat PCR and processing to repeat the evaluation by p53 GeneChip[R].

Sufficient DNA was obtained from 62 breast cancer tissues and twenty-six mutations were identified in 24 of the 62 cases by the p53 GeneChip[R]. No polymorphisms were detected and exon 4 could not be evaluated in 20 cases. There were 43 genetic alterations detected by manual sequencing in 35 of the 62 cases. These consisted of 26 polymorphisms and 17 mutations in exons or splice sites. Fifteen mutations were identified by both methods. Manual sequencing detected significantly more gene alterations (43/54) in DNA extracted from formalin fixed paraffin-embedded (FFPE) tissue than the p53 GeneChip[R] (26/54) (p=0.018). However if the changes in exon 4 were eliminated from this comparison, the p53 GeneChip[R] detected 26 of 27 mutations compared to manual sequencing which identified 16 of 27 mutations (Figure 3) (p=0.016). This suggests that a combination of oligonucleotide microarray and direct sequencing may be necessary to accurately identify p53 gene alterations in formalin fixed paraffin-embedded breast cancer. The p53 GeneChip[R] can not be used to detect polymorphisms in FFPE breast cancer tissue.

Figure 1: p53 polymorphisms identified by PCR-SSCP and sequencing

Representative single strand conformation polymorphism (PCR-SSCP) gels (A,C) and corresponding sequencing gels (B) from representative subjects. Nucleotide change is marked with star. (WT= wild type sequence)

Figure 2: p53 mutations identified by PCR-SSCP and sequencing
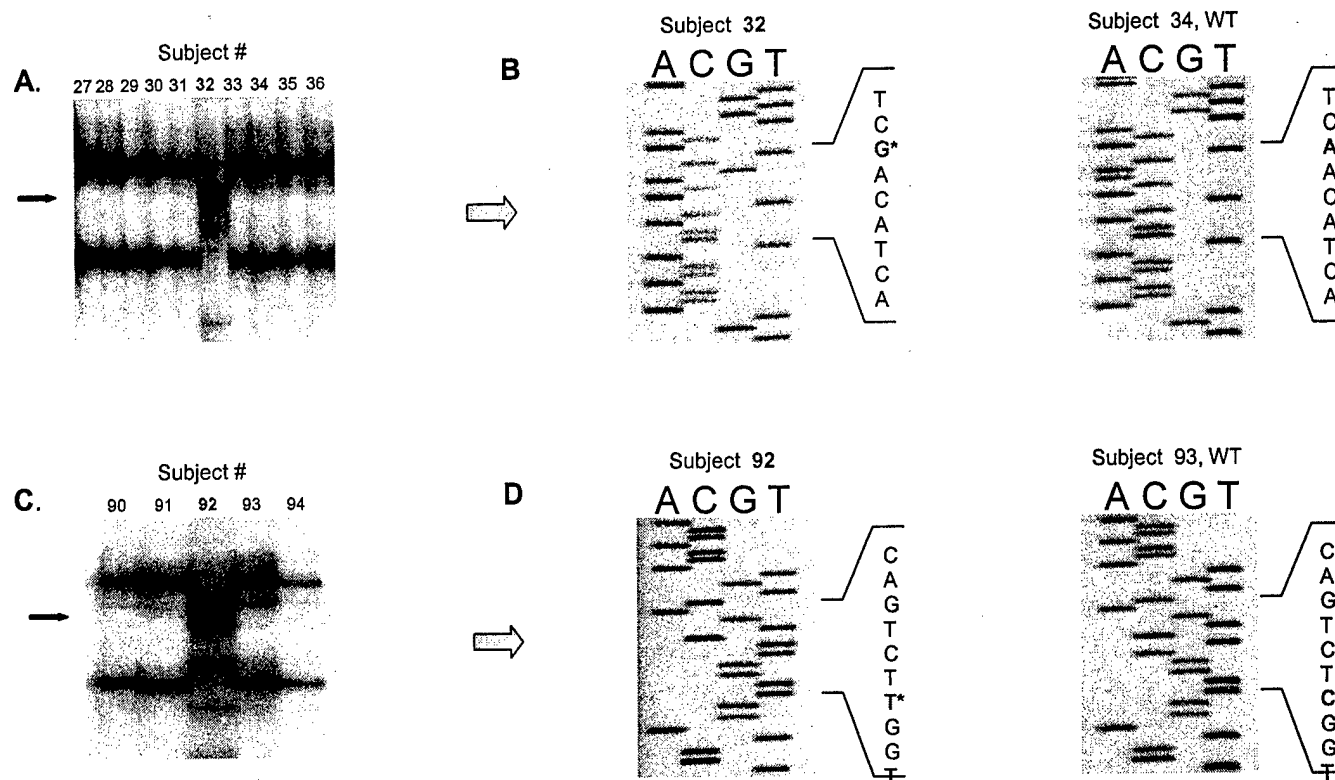
Single strand conformation polymorphism gels (PCR-SSCP) (A,C) and corresponding sequencing gels (B,D) from representative subjects. Nucleotide change is marked with star. (WT= wild type sequence)
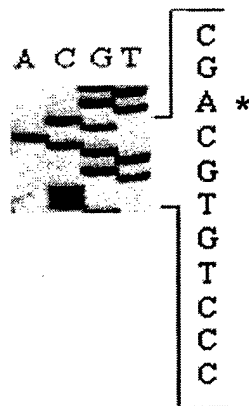
Sequencing gel showing wild type sequence of sample 1.36 for codon 144 (exon 5). The p53 GeneChip^R had showed a mutation in the region indicated

KEY RESEARCH ACCOMPLISHMENTS

1) DNA extracted from formalin fixed paraffin-embedded benign breast tissue is suitable for p53 gene analysis by manual sequencing.

2) DNA extracted from paraffin-embedded tissue can be used for p53 gene analysis using microarray technology (gene chip, Affymetrix). However the DNA must be of good quality. As p53 polymorphisms were not detected using this methodology, it is insufficient on its own to evaluate for p53 alteration at present.

3) As a result of this study an international consortium of researchers was established to investigate biomarkers that will identify women at increased risk to develop breast cancer.

4) P53 gene changes and protein accumulation occur in benign breast tissue and may be a marker to identify women at increased risk to develop breast cancer.

REPORTABLE OUTCOMES

1) Additional grant support:

We received a grant from the Department of Health and Human Services, Public Health Service for grant support for a proposal entitled "p53 in benign breast disease and breast cancer risk: A multicenter Cohort". Tom Rohan is the principal investigator and Rita Kandel is one of the co-investigators. The grant will support the creation of a cohort of over 25,000 women from Portland, Detroit, London (England) and Toronto (Canada) to expand this study of p53 and breast disease and breast cancer risk.

2) Publications:

1) Pollett A, Bedard YC, Li S-Q, Rohan T, Kandel R. Correlation of p53 mutations in thin prep processed fine needle aspirates with surgically resected breast cancer. Mod Pathol 13:1173-79,2000
2) Duffy SW, Rohan TE, Kandel R, Prevost TC, Rice K. Misclassification in a Matched Case-Control Study with Variable Matching Ratio-Application to a Study of c-erbB-2 Overexpression and Breast Cancer. Stat Med 2002. In press.
3) Cooper M, Li SQ, Bhardwaj T, Rohan T, Kandel RA. Evaluation of Oligonucleotide Arrays for Sequencing of the p53 Gene in DNA from Formalin-fixed, Paraffin-embedded Breast Cancer Specimens. Clin Chem., in press 2004 Jan 15
4) Rohan TE, Kandel RA. Breast.. In: Cancer Precursors. Epidemiology, Detection and Prevention. Eds. Franco EL, Rohan TE. pp. 232-248, Springer-Verlag, New York, 2001
5) Kandel RA, Li SQ, Bhardwaj T, Rohan T. p53 Mutation and Breast Cancer Risk. American Association for Cancer Research, Washington,DC; July 2002 (abstract)

3) Manuscript in preparation

Kandel RA, Li S-L, Bhardwaj T, Rohan T. Breast cancer risk and p53 gene change in benign breast disease, in preparation

Individuals who have been employed or paid in whole or part from this grant include:
Melissa Cooper: MSc student
ShuQiu Li: Technician
Tajinder Bhardwaj: Technician
Hanje Chen: Technician
Hangjun Wang: Postdoctoral fellow/research assistant

CONCLUSIONS

1) We are able to extract DNA from the paraffin embedded tissue samples and the DNA is suitable for PCR-SSCP and manual sequencing.

2) Microarray technology, using the p53 Affymetrix gene chip, cannot be used on its own to sequence p53 in DNA extracted from paraffin embedded tissue.

3) Women with benign breast disease who have p53 alteration in an intron which is not a polymorphism and also has p53 protein immunoreactivity in the breast tissue have a 2.9-fold increased risk of developing breast cancer

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Correlation of p53 Mutations in ThinPrep-Processed Fine Needle Breast Aspirates with Surgically Resected Breast Cancers

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Mutations of the p53 gene are one of the most common genetic changes found in cancer; their presence may be prognostic and even influence treatment for breast cancer. In this study, we investigated whether DNA could be extracted from the residual cells left in ThinPrep-processed breast fine-needle aspirates and whether p53 gene changes could be detected in the DNA. The results were then correlated with DNA extracted from the matched formalin-fixed, paraffin-embedded, surgically resected breast cancer when available. DNA was successfully extracted from 54 of 62 aspirates and all 31 surgical specimens. p53 gene mutations were detected in 10 of the 54 cytology specimens (18.5%) and consisted of base pair substitutions or deletions. Silent or intronic p53 changes were found in five additional aspirates. One of the aspirates had two gene alterations, resulting in a total of six gene changes. Five of these changes were located in introns 6 or 9 and the sixth was a silent (no amino acid change) change in exon 6. p53 Polymorphisms were detected in nine aspirates (16.3%) and were located in codon 47 (one aspirate), codon 72 (six aspirates), and codon 213 (two aspirates). All cases with surgical material available showed identical p53 mutations, alterations, and polymorphisms in the resected tumors compared with those detected in the corresponding aspirates. The results of this study show that DNA suitable for analysis of p53 gene sequence changes can be successfully extracted from ThinPrep-processed breast fine-needle aspirates, and that identical alterations are detected in both the cytology and surgical specimens.

Mutations of the p53 gene are among the most common molecular changes detected in human cancers (1). Experimental studies have shown that functional p53 is required for the *in vitro* cytotoxic action of some chemotherapeutic agents (2) The presence of p53 mutations is associated with an increased chemoresistance to doxorubicin in breast cancer patients (3) and may be involved in the development of multidrug resistance (4). Clinical studies have shown that breast cancers that contain p53 gene mutations are associated with decreased disease-free and overall survival (3, 5–9). These results suggest that the presence of p53 mutations might provide prognostic information and influence the treatment of the breast cancer.

Fine-needle aspiration (FNA) of the breast is a safe, effective method for diagnosing breast cancer with minimal intervention and complications (10, 11). As reviewed by Bédard et al., for the detection of carcinoma, it has a sensitivity ranging from 74 to 97% and a specificity ranging from 82 to 100% (12). ThinPrep-processed and conventionally processed breast FNA have been shown to have similar diagnostic accuracy (12). In addition, immunohistochemistry (13, 14) and molecular analysis (15–17) have been successfully applied to ThinPrep-processed specimens.

Because FNA is often the initial sampling of the tumor, it could be a source of cells for the early detection of p53 mutations. In this study, we examined whether p53 mutations could be detected in the cells present in the residual fluid from ThinPrep-processed breast FNAs. When available, the corresponding paraffin-embedded surgically resected tissue was also analyzed for p53 mutations and the results were correlated.

MATERIALS AND METHODS

Specimen Acquisition, Clinical History, and Pathology Review

Cytology reports from November 1997 to April 1999 in the files of Mount Sinai Hospital were reviewed. Of the cases diagnosed as positive or suspicious for malignancy, DNA could be extracted from 54 of 62 specimens of ThinPrep processed breast FNA obtained from 62 different women. In cases in which DNA was successfully extracted from the cytology fluid, the surgical pathology records were reviewed to determine whether there was a corresponding breast tumor specimen. Formalin-fixed, paraffin-embedded tissue was available for 31 women. Clinical details and tumor characteristics were obtained from surgical reports. The breast cancers were graded according to the Elston's modified Bloom and Richardson criteria (18). In 30 of the 31 surgical specimens, the tumor was removed after the cytology specimen. On average, the specimen was removed 33 days after the FNA (range, 8 to 72 days). In one case, the FNA was from a tumor recurrence in the scar 6 weeks after the mastectomy.

p53 Molecular Analysis

DNA Extraction: Cytology

After completing the cytological examination the residual preservative fluid (PreservCyt solution, Cytyc Corporation, Boxborough, MA) was stored at 4°C for up to 3 months. The fluid was centrifuged at 4000 g and the supernatant was removed. DNA was extracted from the remaining cells using TriZol (Gibco-BRL, Rockville, MD). DNA extraction was performed according to the manufacturer's instructions for cells grown in suspension. The DNA was stored at 4°C until used for analysis.

DNA Extraction: Surgical Specimens

Sections (5 μm) were cut from the paraffin blocks and stored for up to 2 weeks. Before microdissec-

tion, the sections were dewaxed and stained briefly with hematoxylin. A representative portion of the tumor containing minimal numbers of stromal and inflammatory cells was microdissected and placed in a microfuge tube. The tissue was digested with proteinase K (0.5 mg/mL in 50 mM Tris·HCl, pH 8.5, 10 mM EDTA, 0.5% Tween 20) for at least 48 hours at 55°C (19). The proteinase K was inactivated by heating at 95°C for 15 minutes. The DNA was stored at −20°C for up to 3 wk until further analyzed.

Polymerase Chain Reaction (PCR)—Single Strand Conformational Polymorphism Analysis (SSCP)

A 1-μL aliquot from each sample was added to 14 μL of PCR solution containing 1.5 mM CaCl$_2$, 20 mM Tris·HCl, pH 8.0, 50 mM KCl, 0.25 μM concentrations of each primer, 0.1 mM concentrations of each dNTP, 1 U Taq DNA polymerase (GibcoBRL, Rockville, MD), and 2 μCi [α-^{33}P]dATP. The primers and the cycling conditions for each exon are listed in Table 1. The reaction product was run on an 8% nondenaturing polyacrylamide gel and the gel was processed for autoradiography (20, 21). Potential mutations were detected by shifts in band mobility. If there was no band shift, the tissue was considered to have no mutation. For samples showing band shifts, the PCR-SSCP analysis was repeated. In cases in which different band shifts were detected in the cytology and corresponding paraffin-embedded samples, an additional paraffin block was selected, cut, microdissected, and processed as above. Negative controls, paraffin-embedded cells that contained no p53 mutation in the exon examined and a water control to replace the DNA, were included in each analysis. Positive controls for exons 5 to 9 (exon 5, SKBr3; exon6, T47D; exon 7, colo 320DM; exon 8, MDA-MB468; exon 9, SW480) were also included where appropriate.

p53 Sequencing

The abnormally shifted band was excised from the SSCP gel and the DNA was eluted into water. The DNA was reamplified by PCR using the same

TABLE 1. p53 PCR Primers and Cycling Conditions

Exon	Primer-sense (5'–3') -antisense (5'–3')	Product Size (bp)	Cycling Parameters
4	ATCTACAGTCCCCCTTGCCG GCAACTGACCGTGCAAGTCA	296	30 cycles; 50 s at 95°C, 50 s at 55°C, 60 s at 72°C
5	GCTGCCGTGTTCCAGTTGCT CCAGCCCTGTCGTCTCTCCA	294	30 cycles; 50 s at 95°C, 50 s at 58°C, 60 s at 72°C
6	GGCCTCTGATTCCTCAGTGA GCCACTGACAACCACCCTTA	199	30 cycles; 50 s at 95°C, 50 s at 55°C, 60 s at 72°C
7	TGCCACAGGTCTCCCCAAGG AGTGTGCAGGGTGGCAAGTG	196	30 cycles; 50 s at 95°C, 50 s at 56°C, 60 s at 72°C
8	CCTTACTGCCTCTTGCTTCT ATAACTGCACCCTTGGTCTC	225	30 cycles; 50 s at 95°C, 50 s at 55°C, 60 s at 72°C
9	GCCTCAGATTCACTTTTATCACC CTTTCCACTTGATAAGAGGTCCC	152	30 cycles; 50 s at 95°C, 50 s at 56°C, 60 s at 72°C

primers and the product was run on a 2% agarose gel. The band was extracted using a QIAquick Gel Extraction Kit (QIAGEN, Chatsworth, CA). The purified DNA was sequenced using a ThermoSequenase radiolabeled terminator cycle sequencing kit (Amersham Life Sciences, Cleveland, Ohio) and the sense primer according to the manufacturer's directions, followed by gel electrophoresis and autoradiography. To confirm the mutation, the DNA product was resequenced using the antisense primer. Negative controls were included in each analysis. Cell lines with known mutations in exons 5 to 9 were also included where appropriate. Mutations were compared with those mutations listed for breast cancer in a known p53 database (http://www.iarc.fr/p53) (22).

Statistical Analysis

The associations between p53 gene alterations and clinical/tumor variables were examined using the χ^2 or, where appropriate, Fisher's exact test (23). Two-sided P-values below 0.05 were considered to be statistically significant.

RESULTS

Histological review of the 31 surgically resected breast tumors showed that they consisted of 29 infiltrating ductal carcinomas not otherwise specified, one invasive ductal carcinoma with lobular features, and one mucinous carcinoma. DNA was successfully extracted from all paraffin-embedded tumors.

Of 62 cytology samples, DNA suitable for p53 sequencing was extracted from 54, yielding an evaluable specimen in 87% of the cases. p53 Gene mutations were detected in 10 of the 54 cytology specimens (18.5%). As shown in Table 2, these consisted of base pair substitutions and deletions. For eight of these 10 aspirates, surgically resected breast tumor tissue was available for gene analysis. All eight cases showed identical p53 mutations in both the aspirate and the surgically resected tumor. A representative SSCP gel is shown in Figure 1 and the associated sequencing gel is shown in Figure 1B.

Other types of p53 gene changes were found in five other aspirates. One aspirate had two gene alterations resulting in a total of six gene changes. As shown in table 3, five changes were located in introns 6 or 9 and one was a silent change (no amino acid change) in exon 6. For two of these five aspirates, surgically resected breast tumor tissue was available for gene analysis and both of the cases showed identical p53 gene changes in the aspirate and the surgically resected tumor.

p53 Polymorphisms were detected in nine aspirates (16.3%) and as shown in Table 4 were located in codon 47 (one aspirate), codon 72 (six aspirates), and codon 213 (two aspirates). For seven of these nine aspirates, surgically resected breast tumor tissue was available for gene analysis and all seven cases showed identical p53 polymorphisms in both the aspirate and the surgically resected tumor.

The clinical features and tumor characteristics were correlated with the p53 gene status and are summarized in Table 5. DNA suitable for p53 sequencing could be obtained from aspirates of tumors of all three grades. The women whose tumors had a p53 mutation or an intronic change or a silent change were grouped together for these analyses because of the small numbers. There was a significant correlation between a younger age ($P = .038$) or larger tumor size ($P = .046$) with the presence of p53 gene alterations. There was no correlation between the presence of estrogen ($P = .449$) or progesterone ($P = 0.066$) receptors or tumor grade ($P = .227$) and the presence of p53 gene alterations.

DISCUSSION

This study demonstrated that DNA can be extracted from ThinPrep processed breast FNAs. This

TABLE 2. Summary of p53 Mutations

Case Number		Exon	Codon	Sequence Change	Amino-Acid Change
Surgical	Cytology				
20	13	5	*	del 23 bases	
9	7	5	130	C→T	Leu→Phe
10	3	5	175	G→A	Arg→His
36	61	5	183	C→G	Ser→STOP
38	29	6	209	del 2 bases	
13	19	6	220	A→C	Tyr→Ser
17	38	7	232	T→G	Ile→Ser
34	60	7	248	G→A	Arg→Gln
NA	59	8	270	T→C	Phe→Leu
NA	62	9	331	C→T	Gln→STOP

*, deletion (del) starting at nucleotide residue 13041 in intron 4 and involving codons in exon 5.
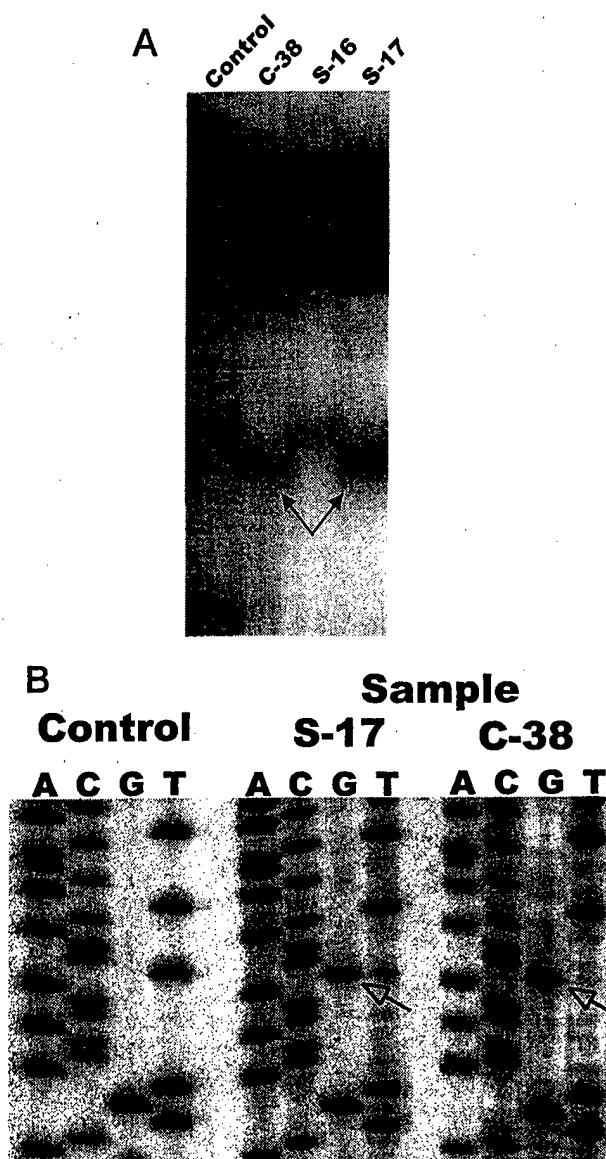NA, tissue not available.

FIGURE 1. A, a representative SSCP gel of p53 exon 7 PCR product from three cases and a negative control (Control). S-16 (surgically resected breast cancer) shows no abnormality. The cytology sample (C-38) and the corresponding paraffin-embedded surgical sample (S-17) show similar band shifts (→). B, the sequencing gel for samples C-38 and S-17 shows a T-to-G base substitution (→). The wild type sequencing pattern (control) in the same region is also shown.

is in keeping with the findings of other groups that have reported successful extraction of RNA or DNA from ThinPrep-processed cytology specimens of breast and cervix (15–17). In addition, the current study showed that the extracted DNA was suitable for p53 gene analysis by PCR-SSCP and sequencing. Using the protocol described above, the mutations detected in exons 4 to 9 were identical to those found in the formalin-fixed, paraffin-embedded, surgically resected breast cancer when this tissue was available for analysis. In contrast, studies assessing p53 immunoreactivity in FNAs and formalin-fixed, paraffin-embedded tumors have shown variable correlations ranging from 73.5 to 93.3% (24–26).

Recent studies have shown that gene alterations detected in paraffin-embedded tissue may be artifacts induced by fixation or processing of surgical specimens (27, 28). Several precautionary steps were undertaken to minimize this possibility. The fidelity of the PCR amplification of DNA extracted from paraffin can be markedly improved by prolonged proteinase K digestion and using small DNA templates (29), so in this study the paraffin-extracted DNA was digested by proteinase K for at least 48 h and the primers were chosen to provide gene sequences of less than 300 base pairs in length. To ensure that the gene alterations were not caused by nucleotide substitutions as a result of *Taq* DNA polymerase misincorporation, all specimens with abnormal SSCP underwent repeat PCR-SSCP to confirm that the change was reproducible. Only those samples that showed similar changes on the repeat PCR-SSCP were considered to have a sequence alteration, which was then confirmed by sequencing. Furthermore, identical alterations were seen in the methanol fixed aspirates and in the corresponding formalin-fixed, paraffin-embedded, surgically resected tumors. This suggests that the p53 alterations identified in this study were genuine.

p53 Mutations were found in 18.5% of patients. This is within the frequency reported for breast carcinoma in other series (8, 9, 30–34). The majority of changes reported for breast cancer have been point mutations (22), and in our series, eight of the 10 mutations (80%) involved base pair substitutions. All mutations, except two (cytology specimens 7 and 13) have been previously reported to occur in breast cancer as listed in a p53 database (22). Silent gene changes were detected in 1.9% of patients, which is similar to the frequency (1.8%) reported by Burns *et al.* (6). In the database examined, there was no report of the silent change observed at codon 224 (cytology specimen 56). No similar comparison could be done for the intronic alterations because the nucleotide position of these types of gene changes is not provided in the database. Codon 47 in exon 4, codon 72 in exon 4 and codon 213 in exon 6 contained known polymorphisms in one, six, and two patients, respectively (1.8, 11.1, and 3.7% of the patients). This is within the range determined for the normal population (35–37). Because the frequencies of mutations and polymorphisms are similar to those shown by others, this suggests that our methodology to detect p53 gene changes is appropriate.

The presence of p53 alterations showed statistically significant associations with larger tumors and younger patient age. No significant association was seen between p53 alterations and tumor grade or the presence or absence of estrogen and progesterone receptors. Other studies examining the associ-

TABLE 3. Summary of p53 Silent and Intronic Changes

| Case Number | | Location | Site | Sequence Change | Amino-Acid Change |
Surgical	Cytology				
NA	56	Exon 6	Codon 224	G→A	Glu→Glu
NA	18	Intron 6	nr 13449	G→C	
NA	55	Intron 6	nr 13964	Del 1 base	
8	35	Intron 6	nr 13964	Del 1 base	
8	35	Intron 9	nr 14755	G→T	
15	5	Intron 9	nr 14766	T→C	

nr, nucleotide residue; NA, tissue not available.

TABLE 4. Summary of p53 Polymorphisms

| Case Number | | Exon | Codon | Sequence Change | Amino-Acid Change |
Surgical	Cytology				
2	36	Exon 4	47	C→T	Pro→Ser
15	5	Exon 4	72	G→C	Arg→Pro
NA	18	Exon 4	72	G→C	Arg→Pro
38	29	Exon 4	72	G→C	Arg→Pro
4	33	Exon 4	72	G→C	Arg→Pro
2	36	Exon 4	72	G→C	Arg→Pro
34	60	Exon 4	72	G→C	Arg→Pro
NA	37	Exon 6	213	A→G	Arg→Arg
31	39	Exon 6	213	A→G	Arg→Arg

NA, tissue not available.

TABLE 5. Patient and Tumor Features

| Features | p53 Status | | P-value |
	Wild-Type	Altered[a]	
Age			
<40	4	2	
40–55	3	7	0.038
56–70	6	1	
>70	7	1	
Tumor Size			
≤2 cm	6	3	
2–5 cm	14	5	0.046
>5 cm	0	3	
Estrogen receptor			
+	13	5	0.449
−	7	6	
Progesterone receptor			
+	13	3	0.066
−	7	8	
Grade			
1	3	0	
2	8	3	0.227
3	9	8	

[a] Altered p53 status includes mutations, silent and intronic changes for surgically resected tumors.

ation between these clinical variables and p53 protein accumulation and/or mutations have yielded inconsistent and often conflicting results. For example, Caleffi et al. found that p53 mutations occurred in younger patients (38) but other studies have not found an association between age and p53 status (5, 39, 40). The number of patients in the current report is small and may have compromised the statistical power of the study to detect associations.

The use of residual cells from ThinPrep-processed samples has several advantages. First, the fluid from ThinPrep processing can be stored at 4°C for up to 3 months, before extracting the DNA, as observed in the present study. Second, because only the residual fluid is needed for analysis, the original diagnostic slides do not have to be used. Third, in contrast to paraffin-embedded tissue, which has to undergo proteinase K digestion for at least 48 h before DNA extraction, ThinPrep-processed aspirates can undergo DNA extraction the same day they are obtained. However, there may also be disadvantages to using the residual material from ThinPrep-processing. Not all cases have tumor cells remaining in the residual fluid and thus DNA may not be available for analysis. In addition, if the aspirate contains numerous benign cells admixed with the malignant cells, mutations may be missed (20, 21).

Immunohistochemical staining can be used to detect p53 protein accumulation in either cytological or surgical specimens (24–26) but the immunohistochemical results do not always reflect the presence of underlying genetic changes (33, 34, 41, 42). For example, nonsense mutations will not cause protein accumulation, so these cells will be negative by immunohistochemical staining. In keeping with this, the presence of p53 mutations in the breast cancer was shown to be associated with decreased disease free survival as well as overall survival (5–9, 31), but the presence of p53 protein detected immunohistochemically in the tumor has not consistently been associated with a worse prognosis (7, 8, 42). As molecular analysis of p53 may provide prognostic and treatment information for patients with breast cancer, ThinPrep aspirate is a

suitable alternative to the paraffin-embedded tissue as a source of cells for this type of analysis in patients who will receive neoadjuvant chemotherapy or have unresectable tumors.

In summary, ThinPrep-processed breast FNAs provide DNA suitable for molecular analysis more rapidly than paraffin-embedded tissue. FNAs seem to be a reliable source of cells to determine the p53 gene status, given that identical alterations were detected in both the cytology and surgical specimens examined in this study.

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Misclassification in a matched case-control study with variable matching ratio: application to a study of c-erbB-2 overexpression and breast cancer

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SUMMARY

We provide a simple analytic correction for risk factor misclassification in a matched case-control study with variable numbers of controls per case. The method is an extension of existing methodology, and involves estimating the corrected proportions of controls and cases in risk factor categories within each matched set. These estimates are then used to calculate the Mantel–Haenszel odds ratio estimate corrected for misclassification. A simulation-based interval estimate is developed. An example is given from a study of risk factors for progression of benign breast disease to breast cancer, in which the risk factor is a biological marker measured with poor sensitivity. Copyright © 2003 John Wiley & Sons, Ltd.

KEY WORDS: matched case-control, misclassification, Mantel–Haenszel estimate; Markov chain Monte Carlo

1. INTRODUCTION

There is a considerable literature on the subject of misclassification of risk factors in epidemiological studies. The various methods are reviewed by Bashir and Duffy [1]. Methods have been developed for use in the settings of the prospective study [2], the unmatched case-control study [3–6] and the pair-matched case-control study [7–9]. In the latter case, Greenland [7, 8] has developed a linear algebraic correction to the estimated numbers of case-control pairs by categories of discrete risk factors, to yield odds ratio estimates which are corrected for the effect of misclassification.

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Table I. Case-control status by c-erbB-2 status, uncorrected for measurement error.

c-erbB-2 status	Number (per cent) of cases	Number (per cent) of controls
Negative	62 (89)	235 (86)
Positive	8 (11)	39 (14)
Total	70	274

To our knowledge, a readily usable method has not been developed for the corresponding problem of a matched case-control study, a binary risk factor and a variable number of controls per case. It is the purpose of this paper to develop such a method, prompted by a case-control study of this design in which we encountered a serious deficiency in sensitivity of detection of the risk factor of interest.

We conducted a matched case-control study of breast cancer nested within a cohort of women with benign breast disease, with the aim of establishing risk factors for progression to cancer [10]. We have 70 cases and a variable number of controls (ranging from 1 to 5) per case. The risk factor under consideration is the immunohistochemical marker c-erbB-2, as determined by NCL-CB11 antibody testing [10, 11]. Uncorrected risk factor status is shown tabulated by case-control status in Table I. The sensitivity of the NCL-CB11 antibody was determined in the study by Press *et al.* [11], in which a group of 187 archival breast cancers with known c-erbB-2 amplification and expression levels was used to characterize the sensitivity of 28 separate anti-c-erbB-2 antibodies. Tissue sections from the tumours were treated with the antibodies, and overexpression was considered to be present when the breast carcinoma cell membranes showed immunostaining. Sensitivity was calculated as (true positives/(true positives + false negatives)).

2. THE PROBLEM

Suppose we have a matched case-control study with m matched sets. Within each matched set l ($l = 1, 2, \ldots, m$), there is one case and n_l controls. Assume we are interested in the effect of a binary risk factor. Within matched set l, let c_l be the proportion of cases with observed risk factor positive (c_l must equal zero or one), and let r_l be the proportion of controls with observed risk factor positive. If there were no misclassification, we could use conditional logistic regression to obtain the odds ratio estimate of relative risk, or equivalently calculate the Mantel–Haenszel estimate stratified by matched set:

$$\text{OR}_{\text{MH}} = \frac{\Sigma(c_l(n_l - n_l r_l))/(n_l + 1)}{\Sigma((1 - c_l)n_l r_l)/(n_l + 1)}$$

Now suppose the determination of the risk factor is subject to error. Clearly, if we perform the statistical analysis using the observed risk factor data, we may obtain biased results [1]. If estimates of the error probabilities are available, there is scope in principle for estimating true risk factor prevalences and deriving an odds ratio estimate which is corrected for the misclassification. Greenland [7–9], develops a correction method whereby the matrix of observed cell counts is multiplied by the inverse of the product matrix of case and control

misclassification probabilities to obtain estimates of the true cell counts. To expand this to the situation of multiple and variable controls per case, it is easier to lay out the calculations in terms of individual cell probabilities rather than in terms of correction by matrix multiplication. Nevertheless, the principle of back-calculation of the true risk factor prevalences within matched sets is essentially the same.

3. CORRECTING THE MANTEL–HAENSZEL ESTIMATE FOR MISCLASSIFICATION

Let $RF = 0$ correspond to risk factor negative status and $RF = 1$ to risk factor positive. Let $OF = 0$ and $OF = 1$ correspond to observed risk factor status. Using Greenland's [7] notation, we let

$$\pi_{ij} = P(OF = i | RF = j)$$

be the error probabilities for the cases and let τ_{ij} be the corresponding error probabilities for the controls. For the case in any matched set

$$P(OF = 1 | case) = P(RF = 1 | case)\pi_{11} + P(RF = 0 | case)\pi_{10}$$

Thus the estimated probability that the case is truly positive for the risk factor may be derived by solution of the above equation for $P(RF = 1 | case)$, as

$$P(RF = 1 | case) = \frac{P(OF = 1 | case) - \pi_{10}}{\pi_{11} - \pi_{10}}$$

Similarly for a control in any given matched set

$$P(RF = 1 | control) = \frac{P(OF = 1 | control) - \tau_{10}}{\tau_{11} - \tau_{10}}$$

The probabilities of being truly risk factor negative are easily calculated by subtraction.

From the above, we can calculate the expected number of cases and controls positive conditional on the observed numbers by substitution of the observed probabilities, and multiplication of the true probabilities by the numbers of cases (invariably 1) and controls (varying from stratum to stratum) in each stratum. Thus the number of cases positive in stratum 1, say, as

$$\frac{c_l - \pi_{10}}{\pi_{11} - \pi_{10}}$$

and the expected number of controls positive as

$$\frac{n_l(r_l - \tau_{10})}{\tau_{11} - \tau_{10}}$$

We can now recalculate the correct Mantel–Haenszel odds ratio estimate using the expected true numbers instead of the observed

$$OR_{MHC} = \frac{\sum_l \frac{(c_l - \pi_{10})(1 - r_l - \tau_{01})n_l}{(\pi_{11} - \pi_{10})(\tau_{00} - \tau_{01})(n_l + 1)}}{\sum_l \frac{(1 - c_l - \pi_{01})(r_l - \tau_{10})n_l}{(\pi_{00} - \pi_{01})(\tau_{11} - \tau_{10})(n_l + 1)}}$$

It should be noted that in the absence of error, this simplifies to the usual Mantel–Haenszel estimate, and in the case of one-to-one matching to Greenland's estimate [7].

We can re-express the numerator of the above as

$$\left\{ \sum_l \frac{n_l c_l(1 - r_l)}{n_l + 1} + \tau_{01}\pi_{10} \sum_l \frac{n_l}{n_l + 1} - \pi_{10} \sum_l \frac{n_l(1 - r_l)}{n_l + 1} - \tau_{01} \sum_l \frac{n_l c_l}{n_l + 1} \right\}$$
$$\times \frac{1}{(\pi_{11} - \pi_{10})(\tau_{00} - \tau_{01})}$$

A similar formula holds for the denominator. Decomposing the summation in this way is useful for computing purposes but has no conceptual value.

4. VARIANCE ESTIMATION AND CONFIDENCE INTERVAL FOR CORRECTED ODDS RATIO

We have gone to some lengths to establish a closed form variance estimate, and tried several forms, but results of simulations indicated that none of our estimates was reliable. We therefore used a simulation technique, Markov chain Monte Carlo [12], to estimate a 95 per cent credible interval on the logarithm of the corrected odds ratio. This is a Bayesian technique, but we specified uninformative priors to give an estimate which was based only on the likelihood. We used the computer package BUGS [12]. The BUGS programme re-expresses the problem as its equivalent conditional likelihood model. The BUGS code is given in the Appendix. The BUGS parameterization and the formulation of the likelihood are as follows.

The data from the validation study are stored in the vectors of and rf in the BUGS programme, so that of[i] is 0 if the observed determination of the risk factor is negative and 1 if it is positive and rf[i] has the same coding for the true status of the risk factor. Both vectors are of length 187, the number of subjects in the validation study for the example.

The data from the case control study are stored in the constants N1–N5 and the matrices Y1–Y5 and of of1–of5. N1 is the number of matched sets with one control, N2 the number with two controls and so on. $YK[i, j]$, where $K = 1, 2, 3, 4$ or 5, records whether person j in set i, with K controls per case, was a case (1) or a control (0). The matrix element ofK[i, j] records whether person j in set i was positive (1) or negative (0) for the observed risk factor. YK and ofK have dimensions NK and $K+1$.

For the likelihood, consider the ith set from the NK sets with K controls. The row $YK[i,] = YK[i, 1], \ldots, YK[i, (K+1)]$ is a vector of zeros and ones with a multinomial distribution with total 1 and probabilities $pK[i,] = pK_1, \ldots pK_{K+1}$ where

$$PK_j = P \text{ (subject } j \text{ is a case} \mid \text{exactly one of subjects } i, \ldots, K+1 \text{ is a case)}$$
$$= \frac{P(j = \text{case} \mid R_j)}{\sum_{m=1}^{K+1} P(m = \text{case} \mid R_m)}$$
$$= \frac{\exp(\beta R_j)}{\sum_{m=1}^{K+1} \exp(\beta R_m)}$$

Table II. True and observed determinations of c-erbB-2 status from the validation study.

Observed determination	True determination	
	Negative	Positive
Negative	118	34
Positive	0	35

In the above, β is the log odds ratio of disease given true risk factor positive as against true risk factor negative. R_j is the true risk factor status, which is unknown, and is represented as rfK[i,j] in the BUGS code. The observed status O_j (ofK[i,j] in the BUGS code) has a Bernoulli distribution with probability $pi[R_j+1]$, where $pi[1]$ is the probability of the observed risk factor being present given that the true risk factor is absent and $pi[2]$ the probability of the observed risk factor being present given that the true risk factor is present. The prior unconditional probability of the true risk factor being present is q, which has a uniform prior between zero and one. This is best illustrated by the code and comments in the BUGS code for the sets with one control per case.

The likelihood of $pi[1]$ and $pi[2]$ are obtained from the external validation study. For each subject in the validation study, the observed response has Bernoulli distribution with probability $pi[1]$ if the risk factor is truly absent and $pi[2]$ if the risk factor is truly present. The likelihood is informative since in the validation study both true and observed status are known. The prior probability for an individual having positive true risk factor status is again q, with a uniform(0,1) prior. Likewise, $pi[1]$ and $pi[2]$ are given uniform(0,1) priors.

5. EXAMPLE

We return to our matched case-control study of progression of benign breast disease to breast cancer and c-erbB-2 status (see Table I). The particular antibody test used for this marker in our study has poor sensitivity, given in a large validation study [11], external to our study population, as 51 per cent. Specificity is quoted as 100 per cent. From these, and from the total number of samples tested in the validation study, 187, with the quoted proportion of 'gold standard' true positives of 39 per cent, we deduce the validation study data in Table II. Assuming non-differential error between cases and controls, this corresponds to $\pi_{00} = \tau_{00} = 1, \pi_{11} = \tau_{11} = 0.51, \pi_{01} = \tau_{01} = 0.49$ and $\pi_{10} = \tau_{10} = 0$.

Results uncorrected and corrected for misclassification are shown in Table III. The correction makes little difference to the point estimate, since although it involves substantial alteration to the estimated prevalence, the alteration applies to both cases and controls. The correction, however, makes a large difference to the interval estimate, since it not only involves correction for the fact that almost half of the true positives are expected to be misclassified, but it also takes into account the fact that the estimated misclassification probability is itself an estimate, with corresponding uncertainty.

Table III. Odds ratios and 95 per cent confidence intervals uncorrected and corrected for measurement error.

Correction	Case prevalence	Control prevalence	OR	95 per cent CI
Uncorrected (100 per cent sensitivity, 100 per cent specificity)	11%	14%	0.72	(0.30, 1.69)
Corrected (51 per cent sensitivity, 100 per cent specificity)	22%	28%	0.66	(0.18, 2.05)

6. DISCUSSION

The method proposed here is a simple adaptation of Greenland's approach [7]. It is easy to obtain the point estimate, although interval estimation requires simulation. The estimated corrected cell frequencies can be expressed as a product of a matrix and a vector, both of which are consistent, so that the consistency of the corrected cell frequency estimates follows in the same way as in Greenland [7]. While the formula for the overall estimate is awkward, its component parts are simple, and it is easy to compute. The BUGS programme in the Appendix gives both a point estimate and a 95 per cent confidence interval. A FORTRAN program for performing the point estimate alone is available from SWD. More general theoretical approaches may be derived as special cases of methods for missing data [13, 14].

When there is 1:1 matching, the point estimate reduces to that of Greenland [7]. In the case of no mismeasurement, the estimate reduces to the usual Mantel–Haenszel estimate. Our interval estimation strategy is dissatisfying in that we have an analytic point estimate and a stochastic interval estimate. Work is in hand to establish a full likelihood solution for both the odds ratio and its variance. In the meantime, we suggest that the MCMC point and interval estimates are used, and that the agreement of the closed form point estimate with that derived from MCMC is used as a check on stability of estimation.

Our example is an interesting one. From Table III, one can see that there is a large correction to the prevalence estimates (if sensitivity is around 50 per cent and specificity 100 per cent, the true prevalence is likely to be around double the observed). One would normally be reluctant to make any use of a measurement which required such a large correction. It is, arguably, justifiable in this case, that of a biomarker measured by a laboratory test with well-documented false positive and negative error rates.

In principle, this method is extendable to the case of multiple levels of a risk factor and/or the effect of several covariates simultaneously. As before, a promising approach would be to build on Greenland's method [7], multiplying a vector of observed proportions in all possible combinations of risk factors by the inverse of a matrix of misclassification probabilities. This is simple in theory, but would give rise to practical problems of dealing with very large matrices if there are numerous potential confounders, and development of variance estimates would be likely to be complex.

In our example, we used both external validation data to calculate the misclassification probabilities. In general, it might be considered preferable to use internal validation, but with three caveats. First, the correction method used here will be inefficient if the validation data allow direct estimation of the predictive values $P(\text{RF} = j | \text{OF} = i)$ [15]. Second, the correction for misclassification is applied multiplicatively, assuming independence of the validation and

the main study. Second, it is frequently the case that internal resources enable only a small validation or repeatability study to be carried out, whereas results of large and therefore more precise independent validation studies may be available from the literature. Perhaps a reasonable strategy is to use information on the misclassification probabilities from both internal and external sources.

APPENDIX: Bugs programme for point and interval estimation

```
model matchcc;

# read in nos of matched sets with 1,2,3,4,5 controls and validation study
size

const N1 = 1,
      N2 = 6,
      N3 = 13,
      N4 = 28
      N5 = 22,
      V = 187;

var Y1[N1,2], of1[N1,2], p1[N1,2], e1[N1,2], rf1[N1,2], rf11[N1,2],
    Y2[N2,3], of2[N2,3], p2[N2,3], e2[N2,3], rf2[N2,3], rf21[N2,3],
    Y3[N3,4], of3[N3,4], p3[N3,4], e3[N3,4], rf3[N3,4], rf31[N3,4],
    Y4[N4,5], of4[N4,5], p4[N4,5], e4[N4,5], rf4[N4,5], rf41[N4,5],
    Y5[N5,6], of5[N5,6], p5[N5,6], e5[N5,6], rf5[N5,6], rf51[N5,6],
    beta, rf[V], of[V], q, pi[2];

data in "/homef/teresa/ccmisc/cont1.sdat",
     in "/homef/teresa/ccmisc/cont2.sdat",
     in "/homef/teresa/ccmisc/cont3.sdat",
     in "/homef/teresa/ccmisc/cont4.sdat",
     in "/homef/teresa/ccmisc/cont5.sdat",
     in "/homef/teresa/ccmisc/valid.sdt";

inits in "/homef/teresa/ccmisc/match2.in";

{
# external validation study
for (i in 1 : V){
      of[i] ~ dbern(pi[rf[i] + 1]);
      rf[i] ~ dbern(q);
}
for (j in 1 : 2){
      pi[j] ~ dunif(0, 1);
}

q ~ dunif(0, 1);
```

```
# matched sets with one control

for (i in 1 : N1){                    # for each set with 1 control per case
        Y1[i,] ~ dmulti(p1[i,], 1);   # multinomial distribution of case status
                                      # with probabilities p1[i,] and sum 1
        for (j in 1 : 2){                       # for each subject in the
matched set
                p1[i,j] <- e1[i,j]/sum(e1[i,]);      # conditional likelihood
                log(e1[i,j]) <- beta*rf1[i,j];       # beta is log(OR)

                rf11[i,j] <- rf1[i,j] + 1;           # distribution of the observed
risk
                of1[i,j] ~ dbern(pi[rf11[i,j]]);     # factor given the true RF
                rf1[i,j] ~ dbern(q);                 # prior on true RF
        }
}


# matched sets with two controls

for (i in 1 : N2){
        Y2[i,] ~ dmulti(p2[i,], 1);

        for (j in 1 : 3){
                p2[i,j] <- e2[i,j]/sum(e2[i,]);
                log(e2[i,j]) <- beta*rf2[i,j];

                rf21[i,j] <- rf2[i,j] + 1;
                of2[i,j] ~ dbern(pi[rf21[i,j]]);
                rf2[i,j] ~ dbern(q);
        }
}


# matched sets with three controls

for (i in 1 : N3){
        Y3[i,] ~ dmulti(p3[i,], 1);

        for (j in 1 : 4){
                p3[i,j] <- e3[i,j]/sum(e3[i,]);
                log(e3[i,j]) <- beta*rf3[i,j];

                rf31[i,j] <- rf3[i,j] + 1;
                of3[i,j] ~ dbern(pi[rf31[i,j]]);
                rf3[i,j] ~ dbern(q);
        }
}
```

```
# matched sets with four controls

for (i in 1 : N4){
        Y4[i,] ~ dmulti(p4[i,],1);

        for (j in 1 : 5){
                p4[i,j] <- e4[i,j]/sum(e4[i,]);
                log(e4[i,j]) <- beta*rf4[i,j];

                rf41[i,j] <- rf4[i,j] + 1;
                of4[i,j] ~ dbern(pi[rf41[i,j]]);
                rf4[i,j] ~ dbern(q);
        }
}

# matched sets with five controls

for (i in 1 : N5){
        Y5[i,] ~ dmulti(p5[i,],1);

        for (j in 1 : 6){
                p5[i,j] <- e5[i,j]/sum(e5[i,]);
                log(e5[i,j]) <- beta*rf5[i,j];

                rf51[i,j] <- rf5[i,j] + 1;
                of5[i,j] ~ dbern(pi[rf51[i,j]]);
                rf5[i,j] ~ dbern(q);
        }
}

beta ~ dnorm(0, 1.0E-4);
}
```

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Molecular Diagnostics
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Evaluation of Oligonucleotide Arrays for Sequencing of the *p53* Gene in DNA from Formalin-fixed, Paraffin-embedded Breast Cancer Specimens

AQ: A

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Background: Routine tissue processing has generated banks of paraffin-embedded tissue that could be used in retrospective cohort studies to study the molecular changes that occur during cancer development. The purpose of this study was to determine whether a *p53* microarray could be used to sequence the *p53* gene in DNA extracted from formalin-fixed, paraffin-embedded (FFPE) tissues.

AQ: B

Methods: DNA was extracted from 70 FFPE breast cancer tissue specimens. *p53* was sequenced with an oligonucleotide microarray (p53 GeneChip®; Affymetrix), and the results were compared with the results obtained from direct sequencing.

Results: DNA was extracted from 62 of 70 cases. We identified 26 mutations in 24 of the 62 cases by the *p53* GeneChip. No polymorphisms were detected, and exon 4 could not be evaluated in 20 cases. There were 43 genetic alterations detected by direct sequencing in 35 of the 62 cases. These consisted of 26 polymorphisms and 17 mutations in exons or splice sites. Fifteen mutations were identified by both methods. Direct sequencing detected significantly more gene alterations (43 of 54) in DNA extracted from FFPE tissue than the *p53* GeneChip (26 of 54; $P = 0.018$). However, if the changes in exon 4 were eliminated from this comparison, the *p53* GeneChip detected 26 of 27 mutations compared with direct sequencing, which identified 16 of 27 mutations. AQ: C ($P = 0.016$).

Conclusions: A combination of oligonucleotide microarray and direct sequencing may be necessary to accurately identify *p53* gene alterations in FFPE breast cancer. The *p53* GeneChip cannot be used to detect exon 4 polymorphisms (codon 72) in FFPE breast cancer tissue.

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Carcinogenesis models suggest that tumor development is caused by the accumulation of mutations in genes that are critical for regulating cell growth *(1–4)*. The genetic alterations that lead to the development of breast cancer have yet to be clearly defined *(5)*, but changes in the *p53* gene are potentially relevant given that it codes for a protein that is involved in regulating the cell cycle, DNA repair, and apoptosis *(6)*. The *p53* gene is one of the commonly altered genes in breast cancer *(7)*, and *p53* mutation rates in breast cancer vary from 15% to 71% depending on the geographic population *(8)*.

Benign breast disease (BBD)[3] is associated with an FN3 increased risk of developing breast cancer *(9, 10)*, and it has been hypothesized that the genetic events that predispose to breast cancer development may also be present in some benign breast lesions. It has been shown that p53 protein can accumulate in BBD such as intraductal hyperplasia with or without atypia, fibroadenomas, fibrocystic disease, and fibrosis *(11–15)* as well as in healthy tissue *(16)* and phylloides tumors *(17)*. Furthermore, p53 protein accumulation appears to be associated with a 2.5-fold

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[3] Nonstandard abbreviations: BBD, benign breast disease; SSCP, single-strand conformation polymorphism; FFPE, formalin-fixed, paraffin-embedded; and IARC, International Agency for Cancer Research.

increased risk of developing breast cancer in women with BBD *(18)*. Mutations in *p53* have also been discovered in healthy and benign breast tissue. One study showed that 2% of cytologically benign fine-needle aspirates had *p53* mutations in the breast epithelium *(15)*. Millikan et al. *(19)* showed that 8% of sporadic forms of BBD have *p53* mutations. Another study using single-strand conformation polymorphism (SSCP) and sequencing analysis examined exons 4–10 of *p53* and observed that 59% (16 of 27) of p53 immunopositive and 27% (4 of 15) of p53 immunonegative healthy breast tissue or tissue with BBD showed genetic alterations in *p53* *(16)*. These results demonstrate that genetic abnormalities can occur in preneoplastic breast lesions. Furthermore, it has been suggested that *p53* alterations may be conserved during the development of breast carcinoma *(20)*.

Retrospective studies using molecular analysis of paired tissue samples from the same patient, such as BBD and breast carcinoma, may help clarify the type and timing of molecular events that occur during breast cancer progression. Routine tissue processing has generated banks of formalin-fixed, paraffin-embedded (FFPE) tissue from patients that can be used in these types of studies. Although the yield of DNA from FFPE tissue is less [four times less than that from fresh tissue *(21)* and 70% of the amount that can be extracted from frozen tissue *(22)* or can be fragmented *(21, 23)*], many of these problems can be circumvented by PCR, which can amplify a small segment of DNA *(23)*, suggesting that this tissue would be suitable for these types of studies.

Direct sequencing is commonly used for detecting *p53* alterations *(24)*, but this method is time-consuming and costly; therefore, other methods of screening for *p53* changes have been developed, such as immunohistochemistry and SSCP analysis. Immunohistochemical detection of p53 protein can underestimate the frequency of *p53* gene changes because not all sequence alterations lead to stabilization of the protein *(25)*. It can also overestimate *p53* gene changes if p53 accumulates for a reason other than mutation of the gene *(26)*. The sensitivity of SSCP is altered by formalin fixation and paraffin embedding and has been shown to have a sensitivity of only 62% in detecting *p53* mutations in DNA extracted from FFPE tissue *(27)* compared with a sensitivity of 90% in DNA extracted from frozen tissue *(28)*.

An alternative method to evaluate the *p53* gene is the *p53* GeneChip® (Affymetrix), which uses oligonucleotide microarray technology to detect mutations *(29)*. The chip contains >50 000 oligonucleotide probes, each of which is 18 nucleotides in length and synthesized by light-directed combinatorial chemistry *(30)*. The probes were created to screen the sense and antisense strands of exons 2–11 for missense mutations, single-base deletions, and the splice sites of the human *p53* coding sequence. The *p53* Gene-Chip has been compared with direct sequencing for identifying *p53* gene alterations in DNA extracted from frozen tissue of 108 ovarian cancers. In one study, the *p53*

GeneChip had a 94% accuracy rate, 92% sensitivity, and 100% specificity compared with 87% accuracy, 82% sensitivity, and 100% specificity for direct sequencing *(31)*. In another study, the *p53* GeneChip was also shown to be comparable to direct sequencing when DNA was extracted from frozen tumor tissue or blood *(32)*. However, its ability to detect *p53* gene alterations in DNA that has been extracted from FFPE tissues is not known. A recent study using arrayed primer extension microarray suggested that it might be possible to assess DNA extracted from FFPE by microarray *(33)*. The purpose of the present study was to determine whether the *p53* GeneChip could be used to sequence the *p53* gene in DNA extracted from FFPE breast cancer tissues. The results were compared with those obtained from direct sequencing.

Materials and Methods

TISSUE ANALYZED

The sequencing and screening methods were performed on paired DNA samples for each case in the study. Seventy breast cancer cases were studied, for which one paraffin block containing breast cancer tissue was selected randomly from the Mt. Sinai Hospital archives for the years 1983–1995. The breast tissue had been fixed in 10% formalin and embedded in paraffin under standard conditions. The diagnosis of breast cancer was confirmed by reviewing representative sections stained with hematoxylin and eosin from each block. All evaluations were done in the absence of any identifying information.

PREPARATION OF DNA

We cut 5-μm sections from the paraffin blocks, which were then dewaxed and stained briefly in hematoxylin. The cancer tissue was microdissected out, collected in a microcentrifuge tube, and digested with proteinase K [Gibco BRL; 0.5 g/L in 50 mmol/L Tris-HCl (pH 8.5), 10 mmol/L EDTA, 5 mL/L Tween 20] for at least 48 h at 55 °C. The proteinase K was inactivated by heating to 95 °C for 15 min. Eight of the 70 cases were discarded because of poor DNA yield. The DNA was then divided in two parts for analysis by direct manual sequencing and oligonucleotide microarray (*p53* GeneChip).

p53 MICROARRAY

Aliquots of DNA were purified with a MiniElute Agarose Gel Purification Kit (Qiagen) according to the manufacturer's protocol. The sample was eluted in 10–15 μL depending on the amount of tissue that had been microdissected. The DNA was amplified in a multiplex PCR as recommended by the manufacturer, and the primers are listed in Table 1. Each 100-μL PCR included 1× PCR buffer (PE Biosystems); 2.5 mM MgCl$_2$ (PE Biosystems); 0.2 mM each of dATP, dCTP, dGTP, and dTTP (Eppendorf); 1× *p53* GeneChip primer set (Affymetrix); and 0.8 U of AmpliTaq Gold (PE Biosystems). The PCR was performed in a PE 9600 thermal cycler. A 5-μL aliquot of the multiplex PCR reaction mixture was visualized on a

AQ: D

AQ: E

AQ: F

T1

Table 1. PCR primers for *p53* GeneChip analysis.

Exon	Primers,[a] 5'–3'	PCR product size, bp
2	TCATGCTGGATCCCCACTTTTCCTCTTG TGGCCTGCCCTTCCAATGGATCCACTCA	162
3	AATTCATGGGACTGACTTTCTGCTCTTGTC TCCAGGTCCCAGCCCAACCCTTGTCC	88
4	GTCCTCTGACTGCTCTTTTCACCCATCTAC GGGATACGGCCAGGCATTGAAGTCTC	366
5	CTTGTGCCCTGACTTTCAACTCTGTCTC TGGGCAACCAGCCCTGTCGTCTCTCCA	270
6	CCAGGCCTCTGATTCCTCACTGATTGCTC GCCACTGACAACCACCCTTAACCCCTC	202
7	GCCTCATCTTGGGCCTGTGTTATCTCC GGCCAGTGTGCAGGGTGGCAAGTGGCTC	173
8	GTAGGACCTGATTTCCTTACTGCCTCTTGC ATAACTGCACCCTTGGTCTCCTCCACCGC	239
9	CACTTTTATCACCTTTCCTTGCCTCTTTCC AACTTTCCACTTGATAAGAGGTCCCAAGAC	144
10	ACTTACTTCTCCCCCTCCTCTGTTGCTGC ATGGAATCCTATGGCTTTCCAACCTAGGAAG	208
11	CATCTCTCCTCCCTGCTTCTGTCTCCTAC CTGACGCACACCTATTGCAAGCAAGGGTTC	223

[a] As provided by the manufacturer.

10% polyacrylamide gel to confirm amplification of 10 PCR products of the correct size. These were then fragmented with DNase I and labeled with fluorescein-dideoxy-CMP. Each 50-μL fragmentation reaction included 45 μL of the multiplex PCR mixture, 0.005 U of fragmentation reagent [DNase I in 10 mmol/L Tris-HCl (pH 7.5), 10 mmol/L CaCl$_2$, 10 mmol/L MgCl$_2$, 500 mL/L glycerol; Affymetrix], 0.03 mmol/L EDTA, 0.05 U of calf intestinal alkaline phosphatase (Roche), and 0.5 mmol/L Tris acetate, pH 8.2. The reaction was incubated for 15 min at 25 °C, followed by heat-inactivation of the enzyme at 95 °C for 10 min. To confirm the fragmentation, a 5-μL aliquot of the sample was visualized in a 2% agarose gel, which showed collapse of the 10 PCR products to fragments of ~50 bp.

Each terminal labeling reaction contained 50 μL of the amplified and fragmented target, 1× reaction buffer, 1× CoCl$_2$, 1× fluorescein-dideoxy-CTP, and 1× terminal deoxynucleotide transferase (all from Enzo Diagnostics). The reaction was incubated at 37 °C for 45 min, and 5 μL of 0.2 mol/L EDTA was added to stop each reaction. To confirm the labeling of the multiplex PCR product, a 3-μL aliquot of the sample was visualized on 2% agarose gel (UVP Gel DocSystem). DNA was hybridized to the *p53* GeneChip, washed, and scanned (GeneChip Microarray Facility, Albert Einstein College of Medicine). Data analysis was performed with the Affymetrix Microarray Suite to generate a score for each sample. A score ≥12 was considered indicative of a gene alteration. When an alteration detected by the *p53* microarray was not confirmed by direct sequencing, the DNA underwent repeat PCR and processing to repeat the evaluation by *p53* GeneChip.

DIRECT MANUAL SEQUENCING

DNA was amplified by use of PCR and primers designed to contain each coding exon of *p53* and the splice sites flanking each exon. The PCR primers (from Eppendorf) and conditions are summarized in Table 2. The 15-μL PCR included 1.5× PCR buffer; 1.5 mM MgCl$_2$ (Qiagen); 1× Q solution (Qiagen); 0.1 mM each of dATP, dCTP, dGTP or dITP, and dTTP (Eppendorf); 0.3 μM the appropriate

Table 2. PCR primers and conditions for direct sequencing of exons 2–11.

Exon	Primers, 5'–3'	PCR product size, bp	Conditions	Number of cycles
2	Forward: CCAGGGTTGGAAGCGTCT; reverse: GACAAGAGCAGAAAGTCAGTCC	260	95 °C for 50 s; 56 °C for 50 s; 72 °C for 60 s	35
3 Partial 4	Forward: ATGGGACTGACTTTCTGC; reverse: CGGCATCTGGACCTGGT	238	95 °C for 50 s; 56 °C for 50 s; 72 °C for 60 s	35
Partial 4	Forward: GGACCATATTCAACAATGGT; reverse: ATGGAACCCAGCCCCTCAG	258	95 °C for 50 s; 56 °C for 50 s; 72 °C for 60 s	35
4	Forward: ATCTACAGTCCCCCTTGCCG; reverse: TGACTTGCACGGTCAGTTGC	345	95 °C for 50 s; 56 °C for 50 s; 72 °C for 60 s	35
5	Forward: GCTGCCGTGTTCCAGTTGCT; reverse: CCAGCCCTGTCGTCTCTCCA	295	95 °C for 50 s; 60 °C for 8 min; 72 °C for 60 s	35
6	Forward: GGCCTCTGATTCCTCACTGA; reverse: GCCAGGACAACCACCCTTA	202	95 °C for 50 s; 56 °C for 50 s; 72 °C for 60 s	35
7	Forward: TGCCACAGGTCTCCCCAAGG; reverse: AGTGTGCAGGGTGGCAAGTG	196	95 °C for 50 s; 56 °C for 50 s; 72 °C for 60 s	35
8	Forward: CCTTACTGCCTCTTGCTTCT; reverse: ATAACTGCACCTTGGTCTC	225	95 °C for 50 s; 56 °C for 50 s; 72 °C for 60 s	35
9	Forward: GCCTCAGATTCACTTTTATCACC; reverse: CTTTCCACTTGATAAGAGGTCCC	152	95 °C for 50 s; 56 °C for 50 s; 72 °C for 60 s	30
10	Forward: TGATCCGTCATAAAGTCAAACAA; reverse: CCCTTACTGGCCCTACTCC	237	95 °C for 50 s; 60 °C for 8 s; 72 °C for 60 s	38
11	Forward: GCACAGACCCTCTCACTCAT; reverse: TGCTTCTGACGCACACCTATTG	272	95 °C for 50 s; 56 °C for 50 s; 72 °C for 60 s	35

forward and reverse primers (Gibco BRL), 0.09 μCi [α-^{33}P]-dATP (Dupont NEN); and 0.07 U of Hotstart Taq (Qiagen) *(16)*. The PCR products were visualized after agarose gel electrophoresis (1% gel; 30 min at 150 V). The amount of DNA was determined by comparing the PCR product with a low-mass DNA ladder (Gibco BRL). Products that were >50 ng were excised and purified by use of the QiaQuick Agarose Gel Extraction Kit (Qiagen). The purified PCR product was sequenced by use of the ThermoSequenase Radiolabelled terminator cycle sequencing reagent set according to the manufacturer's protocol (Amersham Life Sciences). The sequencing reaction PCR was performed using the conditions described in Table 2. After amplification, 4 μL of stop/loading buffer (950 mL/L formamide, 20 mmol/L EDTA, 0.5 g/L bromphenol blue, 0.5 g/L xylene cyanol blue) was added to each reaction, the sample was denatured for 3 min at 95 °C, and 2.5 μL was loaded on a 6% denaturing polyacrylamide gel (8.3 mol/L urea). The gel was processed for autoradiography (Biomax MR film; Kodak). If an alteration was detected, the DNA underwent repeat PCR and sequencing. The product of each sequencing reaction was compared with the *p53* sequence provided by the International Agency for Cancer Research (IARC) database.

AQ: H

The cases in which clear bands were not obtained for exons 5 and 10 were repeated with dITP instead of dGTP. For 22 samples, the exon 4 portion of the exon 3–4 partial PCR product was unreadable. For these samples, PCR and sequencing were performed with primers that were specific for exon 4 only to read the 3′ portion of exon 4 (Table 2). If separation was poor for any of the exons, that exon was sequenced in the reverse direction.

STATISTICAL ANALYSIS

The McMenar test was used to determine whether there were differences between methods in their ability to detect *p53* changes. The resulting test statistics were

referred to a χ^2 distribution ($df = 1$). All statistical analyses were performed with the SigmaStat program. $P \leq 0.05$ was considered statistically significant.

Results

DIRECT SEQUENCING

In the 62 samples analyzed, there were a total of 43 genetic alterations detected by direct sequencing in 35 individuals. These consisted of 26 polymorphisms in 26 individuals and 33 mutations in 17 individuals. An additional 24 alterations were detected in introns, sequences that are not on the microarray and therefore were eliminated from further analysis. Twenty-four of the samples were identified as wild type for all exons. No changes were detected in exons 2, 3, 9, 10, or 11.

Of the 26 polymorphisms, 25 were a G→C change in **AQ: I** exon 4 at codon 72 (Fig. 1), and 1 was an A→G change in exon 6 at codon 213 (see Table 1 in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol50/issue3/). The frequencies of polymorphisms in exons 4 and 6 were 40% (25 of 62) and 2% (1 of 62), respectively.

Mutations were identified in 17 cases, giving a mutation frequency of ~27% (17 of 62). This may be an underestimation because not all exons in all samples could be sequenced for technical reasons (see below). The details of the 17 mutations are summarized in Table 3, **AQ: J** which shows that the changes consisted of single nucleotide base pair changes in 16 of 17 cases. Three individuals had a single nucleotide base pair change at splice sites (intron 5 at nucleotide position 13239, intron 6 at nucleotide position 13432, and intron 7 at nucleotide position 14451). An insertion was detected in one case. Eight cases had a polymorphism in exon 4 as well as a mutation elsewhere.

No PCR product could be obtained for six samples for exon 2, eight samples for exon 3, and one sample for exon 11. Eleven changes in exon 2 and 8 changes in exon
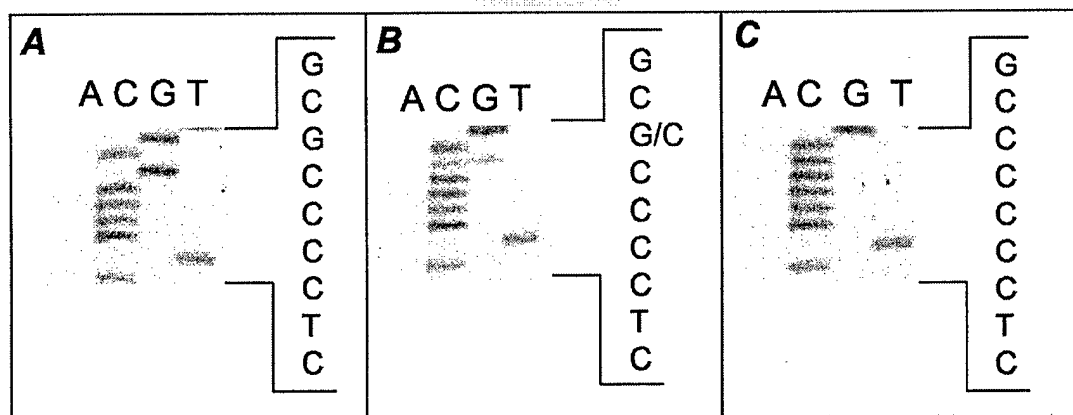

Fig. 1. Sequencing gels showing samples that are wild type (*A*) or have the G→C nucleotide change (*B* and *C*) indicative of the polymorphism at codon 72 (exon 4).

The sample in *C* is homozygous for this polymorphism.

Table 3. *p53* mutations.

Sample	Location	Site	Sequence change	Amino acid change	Type of mutation	Method of detection[a]
1.27	Exon 5	Codon 155	ACC→AAC	Thr→Asn	Missense	C, S
1.4	Exon 5	Codon 175	CGC→CAC	Arg→His	Missense	C, S
1.9	Exon 5	Codon 179	CAT→GAT	His→Asp	Missense	C, S
1.17	Exon 6	Codon 214	CAT→CGT	His→Arg	Missense	C, S
2.46	Exon 6	Codon 220	TAT→TGT	Tyr→Cys	Missense	C, S
2.45	Exon 7	Codon 248	CGG→CAG	Arg→Gln	Missense	C, S
2.3	Exon 8	Codon 271	GAG→AAG	Glu (Gln)→Lys	Missense	C, S
2.56	Exon 8	Codon 273	CGT→TGT	Arg→Cys	Missense	C, S
2.36	Exon 8	Codon 282	CGG→TGG	Arg→Trp	Missense	C, S
1.15	Exon 8	Codon 284	ACA→CCA	Thr→Pro	Missense	C, S
1.1	Exon 8	Codon 306	CGA→TGA	Arg→Stop	Nonsense	C, S
2.15	Exon 8	Codon 306	CGA→TGA	Arg→Stop	Nonsense	C, S
1.24	Intron 5	nt[b] 13239	G→C		Splice site	C, S
2.13	Intron 6	nt 13432	G→A		Splice site	C, S
1.25	Intron 7	nt 14451	G→T		Splice site	C, S
1.36	Exon 5	Codon 144	CAG→CTG	Gln→Leu	Missense	C
1.38	Exon 5	Codon 144	CAG→CTG	Gln→Leu	Missense	C
1.41	Exon 5	Codon 160	ATG→ATC	Met→Leu	Missense	C
2.60	Exon 5	Codon 144	CAG→CTG	Gln→Leu	Missense	C
1.25	Exon 5	Codon 144	CAG→CTG	Gln→Leu	Missense	C
2.18	Exon 5	Codon 167	CAG→GAG	Gln→Glu (Gln)	Missense	C
2.56	Exon 6	Codon 194	CTT→TTT	Leu→Phe	Missense	C
1.11	Exon 7	nt 14075, 14076	C→del		2-bp deletion	C
2.1	Exon 7	Codon 249	AGG→GGG	Arg→Gly	Missense	C
2.57	Exon 8	nt 14480	G→del		Deletion	C
2.23	Exon 8	Codon 272	GTG→AGA	Val→Arg	Missense	C
1.5	Exon 6	nt 13397	G		Insertion	S
2.18	Exon 4	Codon 89	CCC→CCT	Pro	Silent	S

[a] The method by which the *p53* mutation was detected: C, *p53* GeneChip analysis; S, direct sequencing.
[b] nt, nucleotide; del, deletion.

3/portion of exon 4 could not be confirmed because after multiple attempts no repeat PCR product could be generated. These samples were not included in the analysis for these exons. There were 12 changes that were identified by the first round of PCR and direct sequencing that were not confirmed in the repeat analysis, and these cases were considered negative.

p53 MICROARRAY

A total of 26 genetic alterations were identified in 24 cases by the *p53* GeneChip, giving a mutation frequency of 40% (24 of 62; Table 3). There were 22 cases with one change only and 2 cases with two changes. A 2-bp deletion was detected for one case (1.11) as indicated by a single deletion at two contiguous nucleotides, each with a score of 13. Fifteen of the 26 changes were also detected by direct sequencing. Thirty-seven samples were identified as wild type for all exons. No polymorphisms were detected. There were no changes detected in exons 2, 3, 4, 9, 10, or 11.

A total of 21 samples that had selected exons could not be analyzed for technical reasons. These included exon 4 (20 cases), exon 5 (4 cases), exon 9 (2 cases), intron 10 (4 cases), exon 10 (2 cases), and exon 11 (3 cases). Twenty-

four changes could not be confirmed on repeat analysis, and these were considered wild type.

Microarray analysis failed to detect two mutations identified by direct sequencing; an insertion in exon 6 and a single base pair change in exon 4. In the latter case, exon 4 did not amplify sufficiently and therefore could not be analyzed by the *p53* GeneChip; it thus is not a true negative.

COMPARISON OF DIRECT SEQUENCING AND *p53* GeneChip RESULTS

There were nine missense mutations and two deletions identified and confirmed by the *p53* GeneChip that were not identified by direct sequencing. The sequencing gels were re-reviewed for these 11 cases. In one case the change had been missed initially because of high background. Although this change could be seen in retrospect, it was not included as a positive case in the data analysis because background is a factor in interpreting direct sequencing gels. The remaining 10 mutations still could not be detected. For these cases, the PCR and direct sequencing were repeated again, and all but two cases were still negative. The two cases that became positive on repeat analysis showed an abnormal sequence pattern at

the same position as identified by the microarray (codon

249, exon 7). In the other case (single base pair change at codon 144 in exon 5; Fig. 2), the change would have been considered background without the *p53* GeneChip information and therefore was still considered a negative for the statistical analysis.

The ability to detect different types of mutations varied between the two sequencing methods. The *p53* GeneChip showed a higher mutation detection rate for missense mutations (100%; 19 of 19) than direct sequencing (52%; 10 of 19). Although the numbers are small, the two sequencing methods were able to identify other types of mutations equally well. This included nonsense and splice junction mutations with mutation detection rates of 100% (2 of 2 and 3 of 3, respectively) for direct sequencing and the *p53* GeneChip.

The ability of each method to identify changes in each exon/intron was calculated by determining the number of alterations detected by the individual method relative to the combined number of confirmed alterations detected by direct sequencing and the *p53* GeneChip. This approach was taken because each method appeared to identify some different alterations. Direct sequencing detected 80% of total changes (43 of a total of 54 possible alterations), and the *p53* GeneChip detected 48% (26 of a total of 54 possible alterations). To determine whether oligonucleotide microarray analysis or direct sequencing is better at identifying *p53* alterations, the proportion of cases that showed alterations by each method was calculated together with the corresponding 95% confidence intervals. Direct sequencing was significantly better than the chip in detecting all alterations ($P = 0.018$). However, if exon 4 results were eliminated from the analysis because the PCR product for this exon could not always be amplified, then the *p53* GeneChip detected more mutations (26 of 27 mutations) than direct sequencing (16 of 27 mutations; $P = 0.016$).
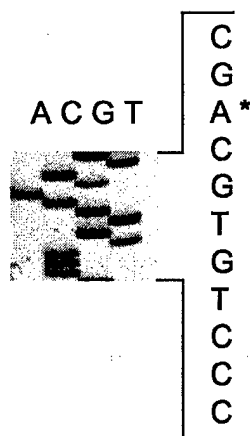

Fig. 2. Sequencing gel showing the wild-type sequence of sample 1.36 for codon 144 (exon 5).

The *p53* GeneChip had showed a mutation in the region, indicated by * (CAG→CTG).

Discussion

In this study, we showed that DNA extracted from FFPE tissue can be used for *p53* GeneChip analysis. Under our conditions, direct sequencing detected significantly more gene alterations than the *p53* GeneChip. This difference could be attributed in part to the difficulty in reliably amplifying exon 4 with the primers provided with the GeneChip. The product generated for this exon is the largest (366 bp) of the 10 PCR products and because of its size was frequently not successfully amplified (20 of 62 cases) and limited the changes that could be detected by the *p53* GeneChip for this exon. This was not a problem for direct sequencing because we designed multiple primer sets to allow for amplification of smaller fragments of exon 4, which appeared to eliminate the problem of amplifying a single large product from DNA extracted from FFPE. This can not be done for the microarray because the system is optimized for a multiplex reaction. When the results for exon 4 were excluded from the analysis, the *p53* GeneChip detected 37% more alterations, most of which were missense mutations, than direct sequencing which is an important finding when choosing the optimum method for *p53* gene analysis. This observation is in keeping with the results of other studies, which showed that oligonucleotide microarray evaluation of the *p53* gene in tissue that were not formalin-fixed and paraffin-embedded had a sensitivity that was equal to or better than that of direct sequencing (*31, 32*). There are several reasons for the equivalent or superior performance of microarray evaluation (after exclusion of exon 4). One reason is that the array analysis may be less affected than direct sequencing by the fragmentation of DNA caused by the processing of the tissue because the PCR products undergo a step in which the product is reduced to 50 bp in preparation for the *p53* GeneChip analysis. Another reason is that it is possible that we did not design the optimum primers for direct sequencing that would identify all abnormalities because this likely would require multiple primer sets and an unlimited supply of DNA. A third reason is that it has been shown that direct sequencing can only detect a mutation when at least 30% of the total DNA is mutant (*34*), whereas the *p53* GeneChip can detect mutations if <2% of cells are altered (*35*). Finally, structural reasons might also explain why some alterations were not detected by direct sequencing. DNA sequences containing GC-rich regions may not denature completely during electrophoresis because of the strong hydrogen bonds that form between these two nucleotides. Incomplete denaturing of the strands can disturb the migration pattern of the DNA fragments and cause bands to compress. Although dGTP was replaced with dITP, a nucleotide analog that forms weaker bonds with dCTP than dGTP, this substitution can lead to other problems. For example, one consequence of using dITP is that it may impair the ability of direct sequencing to identify heterozygous changes because dITP gives less uniform band intensities (*36*). Because one-half of the

changes missed by direct sequencing were in exon 5, the exon for which dITP was used most frequently, it is possible that it compromised our ability to identify changes in small populations of cells.

Other factors should be considered when comparing these two methods. The GeneChip cannot detect intronic changes outside of the splice sites, and this could be a limitation in a study in which it is necessary to evaluate intronic DNA sequences. The GeneChip also cannot detect insertions and large deletions. In addition, use of the arrays is costly. However, the *p53* GeneChip analysis takes approximately one-fifth of the time that it takes to perform direct sequencing, giving cost savings through a reduced need for personnel.

The *p53* GeneChip analysis and direct sequencing had similar results in terms of the number of nonreproducible changes. No similarities were seen in the nonreproducible changes identified by either sequencing method. False positives are expected because both sequencing methods are PCR based and changes that can not be confirmed are most likely attributable to PCR artifacts. *Taq* polymerase has been shown to misincorporate 1 bp in every 10 000 bp *(37)* or as many as 1 in 500 bp in DNA extracted from small amounts of FFPE tissues *(38)*. Our results would suggest that, similar to direct sequencing, all samples considered to have a gene alteration by microarray analysis should undergo repeat PCR and GeneChip analysis.

An unexpected limitation identified in this study was the inability of the oligonucleotide microarray to detect polymorphisms. There were 26 polymorphisms identified by sequencing. Of these, 13 were present in samples for which exon 4 could not be analyzed. In the case with a polymorphism in exon 6, the *p53* GeneChip had a score of 6, which according to our cutoff criteria indicated a negative result. However, if there was alternative tiling for this change present on the chip, our cutoff score may have been too high (personal communication from Affymetrix). It has been suggested by Wikman et al. *(35)* that fixed cutoff scores may not be appropriate and that the amount of background present for each probe should be factored into the determination of the cutoff value rather than having a single score for all exons. However, it is not clear why in the 12 cases in which exon 4 could be examined the polymorphisms were not detected by the *p53* GeneChip. This is a particularly unexpected finding because the polymorphism would be present in at least 50% of the cells if it were a heterozygous change. It is possible that the secondary structure of the gene in this region affects the ability of the DNA to hybridize to the chip *(39)*. Because exon 4 has a 78% G:C content and the region in and around codon 72 is GC rich, the formation of stable secondary structures may explain why the *p53* GeneChip did not detect this polymorphism under our conditions. Another study has reported detection of polymorphisms by the oligonucleotide microarray *(31)*, but in that study the DNA was extracted from frozen tissue and a lower score was used to indicate the presence of an alteration. The authors did not state which polymorphisms were detected, and it is possible that the polymorphisms were different because the authors evaluated ovarian cancers and not breast cancers.

The chip identified four samples with a mutation in codon 144 that produced a glutamine-to-leucine change. This change is present in the p53 database but has only been detected in lymphoma and cancers of the prostate, brain, and pancreas. This mutation was not identified by direct sequencing in any of the cases, perhaps because it was present in too few of the cells. Alternatively, it is possible that it represents an artifact of the chip detection method.

It could be argued that direct sequencing of DNA extracted from frozen (or fresh) tissue should be the standard against which DNA extracted from FFPE tissue and analyzed by *p53* GeneChip should be evaluated. It may be that the use of FFPE tissue in this analysis generated false positives and negatives, a particular problem when the amount of tissue to be analyzed is limited *(38, 40)* However, the purpose of this study was to evaluate these methodologies in this type of processed tissue. Furthermore, there have been several studies suggesting that in practice, artifacts introduced by FFPE may not be a major problem because similar genetic alterations were detected in DNA extracted from FFPE tissue compared with DNA extracted from frozen tissue from the same tumor *(41–45)*. Furthermore, all of the exonic mutations identified in this study were in the database of *p53* mutations maintained by the IARC (www.iarc.fr/p53). Direct sequencing detected mutations in ~27% of cases, a finding in keeping with the frequencies of *p53* mutations in breast cancer that have been reported, which vary between 15% and 71% *(8)*. The IARC database shows that there is a wide range of frequencies for polymorphisms at codon 72, and the frequency of this polymorphism in the current study was within this range *(46)*. Similarly, the frequency of the codon 213 polymorphism is up to 11% in this database, and our results were in keeping with this. The absence in these cases of insertions/large deletions, changes that are known to occur in breast cancer, may reflect the detection method, but it is more likely that the absence of this change is related to the size of the study. The proportion of *p53* mutations in breast cancer that are deletions and insertions is relatively low, 8% and 3%, respectively *(47)*.

Furthermore, several technical precautionary steps were taken in the current study to minimize the possibility of over- and/or undercalling of sequence abnormalities. Every case with a possible alteration was confirmed by a repeat PCR, and it is very unlikely that the same artifact would occur in two separate reactions. In addition, it has been shown that if the tissue is digested sufficiently and the products are small, this will minimize PCR artifacts *(48)*. In this study, all PCR products were <300 bp in length except for those for exon 4.

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In conclusion, direct sequencing detects more alterations in DNA extracted from FFPE breast cancer tissue than the *p53* GeneChip. However, if exon 4 is eliminated from the evaluation, the *p53* GeneChip detects significantly more mutations than direct sequencing. At present it would seem most appropriate to screen the *p53* gene with the *p53* GeneChip. In those cases in which exon 4 can not be evaluated by the GeneChip or in which there is a need to sequence introns, these can be done by direct sequencing. The microarray cannot be used to detect exon 4 polymorphisms (codon 72) in FFPE breast cancer tissue.

AQ: N

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